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Award Number: W81XWh-05-1-0250

TITLE: Interaction of AIB1 and BRCA1 in the Development of Breast Cancer

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REPORT DATE: March 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-03-2006	Annual Summary	21 Feb 2005 – 20 Feb 2006

4. TITLE AND SUBTITLE

Interaction of AlB1 and BRCA1 in the Development of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0250

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

John T Lahusen

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

AIB1 (SRC3) belongs to the p160 family of steroid receptor coactivators including SRC-1 and SRC-2. AIB1 interacts with several nuclear receptors including estrogen and progesterone receptors in a ligand-dependent manner and enhances their transcriptional activity. AIB1 is amplified and overexpressed in >30% of breast cancers and can increase the sensitivity of breast cancer cells to estrogen and to growth factor signaling. BRCA1 regulates cell cycle progression, apoptosis induction, transcription, and DNA repair. From 5-10% of total breast cancers are due to germ-line BRCA1 mutations that lead to a deficiency in the BRCA1 protein. We have observed that AIB1 can partially reverse BRCA1 mediated repression of ER-dependent transcriptional activity in breast cancer. This research will identify if there is a functional consequence of an interaction between AIB1 and BRCA1 in breast cancer.

15. SUBJECT TERMS
Breast Cancer

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	19	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The *AIB1* gene was initially identified to be amplified on a portion of human chromosome 20q that is frequently amplified in breast cancer[1]. AIB1 (SRC-3) was later characterized as a nuclear receptor coactivator that belongs to the p160 family of steroid receptor coactivators including SRC-1 and SRC-2. Steroid receptor coactivators recruit other coactivators and the basal transcriptional machinery to nuclear receptors. AIB1 interacts with nuclear receptors including estrogen and progesterone receptors in ligand-dependent manner and enhances their transcriptional activity. The *AIB1* gene is amplified in 5-10% of breast cancers and both the mRNA and protein are overexpressed in >40% of breast tumors and breast cancer cell lines[2-4]. An isoform of AIB1, AIB1-Δ3, encodes a 130 kDa protein that lacks the amino-terminal bHLH/PAS dimerization domain and is a more active transcriptional coactivator of ERα and PR than full-length AIB1[5]. AIB1 and AIB1-Δ3 also affect growth factor signaling pathways including IGF-I and EGF[6-8].

Germ-line mutations of the breast cancer susceptibility gene *BRCA1* account for 5-10% of breast cancers. BRCA1 is involved in DNA repair, progression through the cell cycle, apoptosis, maintenance of DNA integrity, and regulation of transcription. BRCA1 has been shown to physically associate with transcriptional activators, repressors, DNA-binding transcription factors, and DNA repair factors. It has been reported that BRCA1 represses the transcriptional activity of estrogen and progesterone receptor. The lack of wild-type *BRCA1* expression in mammary epithelium likely leads to increased DNA damage and cellular proliferation. BRCA1 may modulate the over-proliferation of mammary epithelial cells by estrogen. The loss of the inhibitory activity of BRCA1 may lead to mammary carcinogenesis. It has been reported that BRCA1 interacts with SRC-1[9], but there are no reports of an interaction of AIB1 (SRC3) with BRCA1. Preliminary data in our lab showed that AIB1 and BRCA1 protein

interact in MCF-7 breast cancer cells. As it has been reported that BRCA1 represses ERα activity in breast cancer cells, I assessed whether overexpression of AIB1 could reverse this effect. Also, preliminary evidence suggests that AIB1 can partially reverse the BRCA1-induced repression of ERα transcriptional activity**.** We propose that the ratio of BRCA1 and AIB1 in the cell determines the response to estrogen. It could be that high BRCA1 levels in the cell leads to inhibition of AIB1's coactivator ability and decreased ERα activity, but when AIB1 is overexpressed or levels of BRCA1 decrease then AIB1 is free to coactivate ERα transcriptional activity. Increased estrogen-induced gene expression can lead to over-proliferation of breast epithelial tissue and eventually tumorigenesis. I want to determine if there is a functional consequence of AIB1 and BRCA1 in breast cancer in relation to hormone and growth factor signaling.

BODY

This annual report will address the progress for fulfilling the aims as outlined in **Task 1** of the **Statement of Work** for grant W81XWh-05-1-0250. Section (a) of **Task 1** is to determine if AIB1 and BRCA1 protein interact directly in breast cancer cells and then define the region/s necessary for interaction with BRCA1. Preliminary experiments showed that AIB1 and BRCA1 protein interacted by co-immunoprecipitation using MCF-7 cellular lysate (Figure 1). In this experiment, BRCA1 was immunoprecipitated with a BRCA1 antibody (Ab2, Oncogene Science) and then an immunoblot was done with an AIB1 antibody (BD Transduction). There was increased association of AIB1 with BRCA1 in this experiment. Also, the reverse immunoprecipitation was done with the same AIB1 antibody and then immunoblotted with the BRCA1 antibody (Figure 2). In this experiment, MCF-7 cells were either transfected with empty vector (pcDNA3.1) or with a BRCA1 plasmid and cell lysate was used for the

immunoprecipitation. MCF-7 cells endogenously express BRCA1 but I wanted to determine if increased BRCA1 expression resulted in increased association with AIB1. There was increased association of BRCA1 with AIB1 in this experiment but overexpressing BRCA1 did not increase the interaction of AIB1 and BRCA1. Unexpectedly, increased BRCA1 expression resulted in reduced association between AIB1 and BRCA1. To verify the interaction between AIB1 and BRCA1 I also performed immunoprecipitations with a mouse immunoglobin (Figure 3). The results from this experiment suggest that AIB1 is binding in the mouse immunoglobulin control. I need to modify the conditions for the immunoprecipitation to reduce the non-specific binding of AIB1 to conclude further about the interaction of AIB1 with BRCA1. I used gamma-bind sepharose beads (Amersham Bioscience) to capture the antibody complexes. I plan to block the gamma sepharose beads with 0.5% bovine serum albumin to determine if this eliminates non-specific binding of proteins in the immunoprecipitation.

Also, to further study the interaction of AIB1 with BRCA1 I have been able to obtain AIB1-GST (glutathione-s-transferase) constructs that cover the entire length of AIB (Figure 4)[10]. I currently need to develop a full-length AIB1-GST construct by sub-cloning the AIB1 cDNA into the pGEX prokaryotic expression vector. Our lab is also using the mammalian-two-hybrid system to study protein-protein interaction which could be used to analyze the interaction of AIB1 with BRCA1. We have developed an AIB1-VP16 construct to be used in this assay. I would need to develop the BRCA1-GAL4 construct or obtain it from another lab. The vectors would be transfected into cells along with the GAL4-luciferase reporter. If AIB1 and BRCA1 interact, this would result in activation of the promoter and production of luciferase protein. Luciferase levels would be quantified with a luminometer.

Section (b) of **Task 1** is to determine if overexpression of BRCA1 in breast cancer cells is able to suppress the coactivator potential of AIB1 on estrogen receptor transcriptional activity but is unable to reverse the coactivation potential of a mutant AIB1 that does not bind to

BRCA1, AIB1(BRCA1-). BRCA1 has been shown to suppress estrogen-dependent transcription in breast cancer cells[11]. As part of my **Statement of Work**, I wanted to duplicate the results for BRCA1 repression of estrogen-dependent transcription and to determine if AIB1 can reverse BRCA1 repression of ER transcriptional activity. Preliminary data from MCF-7 and T47D breast cancer cells indicated that BRCA1 can suppress estrogen-stimulated estrogen receptor transcriptional activity (Figure 5). AIB1 was able to partially reverse the repression of ER transcriptional activity by BRCA1 in MCF-7 cells but not in T47D cells. I did not observe enhanced activation of ER transcriptional activity with AIB1 in these experiments as has been observed in other cell lines. Our lab has published that AIB1 and an amino-terminally deleted isoform of AIB1, AIB1-Δ3, coactivates both ER and PR-dependent transcriptional activity using Chinese hamster ovary cells as a model system[5]. I have observed that AIB1 and AIB1-Δ3 also coactivate ER reporter assays using MCF-7 cells but the results are variable. MCF-7 cells express high endogenous levels of AIB1 protein due to amplification of the gene so it is more difficult to observe coactivation in transient ER transcriptional reporter assays. I am testing other breast cancer cell lines to determine if there is a better model system for ER transcriptional assays. I also used T47D cells which have lower levels of AIB1 protein expression than MCF-7, but I did not observe coactivation of an ER-dependent transcriptional reporter. This could be due to competition with other steroid receptor coactivators in the transcriptional complex.

For **Task 2**, I wanted to address the role of AIB1 and specifically AIB1 (BRCA1-) on IGF-1 and EGF induced phenotypic effects and changes in gene expression inependently of estrogen. I tested HCC1937 (BRCA1-deficient) and MCF-7 (BRCA1-wild type) breast cancer cells for their response to growth factor-induced cyclin D1 promoter activity. Our lab obtained the -1745 cyclin D1 promoter inserted into the PA3-luciferase plasmid[12]. I transfected the MCF-7 cells with the cyclin D1 promoter construct and then stimulated with EGF, IGF-1 and heregulin-β. Heregulin β is the ligand for ErbB3, a member of the EGFR family of receptors,

which causes ErbB3 to heterodimerize with ErbB2. It has been reported that heregulin β induces the transcription of cyclin D1 in the T47D breast cancer cell line[13]. I observed that EGF, IGF-1 and heregulin β could induce the activity of the cyclin D1 promoter 2.6-, 1.9-, and 11-fold respectively in MCF-7 cells (Figure 6). Heregulin β also increased the activity of the cyclin D1 promoter in HCC1937 cells about 2-fold (Figure 7). From these results, it seems the cyclin D1 promoter is more responsive to EGF and heregulin β stimulation than IGF-1 stimulation. I also analyzed whether AIB1-Δ3 had any role in heregulin β-induced cyclin D1 promoter activity. I observed that AIB1-Δ3 enhanced growth factor-dependent and -independent cyclin D1 promoter activity in HCC1937 cells. Experiments to determine if BRCA1 modulates the AIB1 response are in progress. I am currently in the process of developing HCC1937 (BRCA1-deficient) cells that stably express BRCA1.

Figure 1

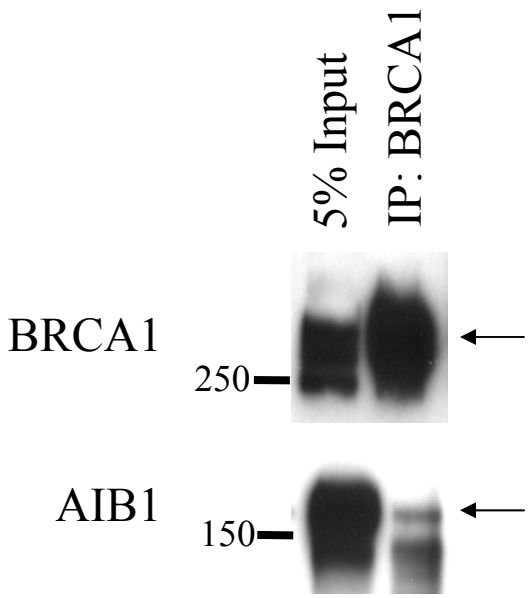


Figure 1. **Co-immunoprecipitation of AIB1 with BRCA1 in MCF-7 breast cancer cells**. Whole-cell extract was immunoprecipitated with a monoclonal BRCA1 antibody (AB2, Oncogene Science) and then an immunoblot was done with a monoclonal AIB1 antibody (BD Transduction).

Figure 2

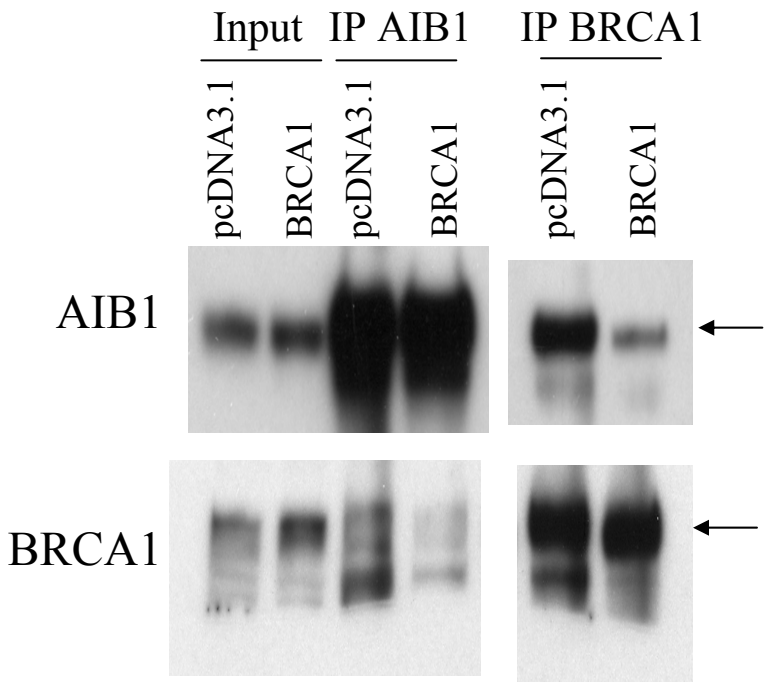


Figure 2. **Co-immunoprecipitation of AIB1 with BRCA1 after transfection of BRCA1 in MCF-7 breast cancer cells.** MCF-7 cells were transfected with either pcDNA3.1 plasmid or BRCA1 plasmid using Fugene 6 (Roche). Whole-cell extract was immunoprecipitated with either a monoclonal BRCA1 antibody (AB2, Oncogene Science) or monoclonal AIB1 antibody (BD Transduction) and then immunoblotted with either AIB1 or BRCA1.

Figure 3

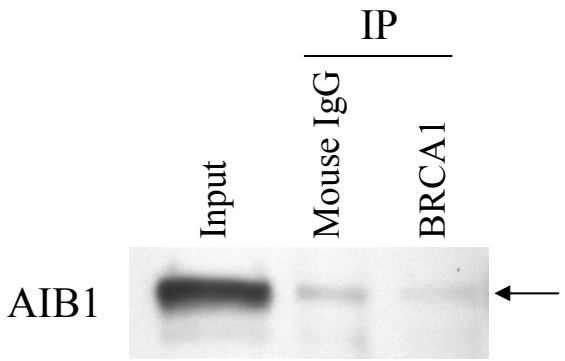
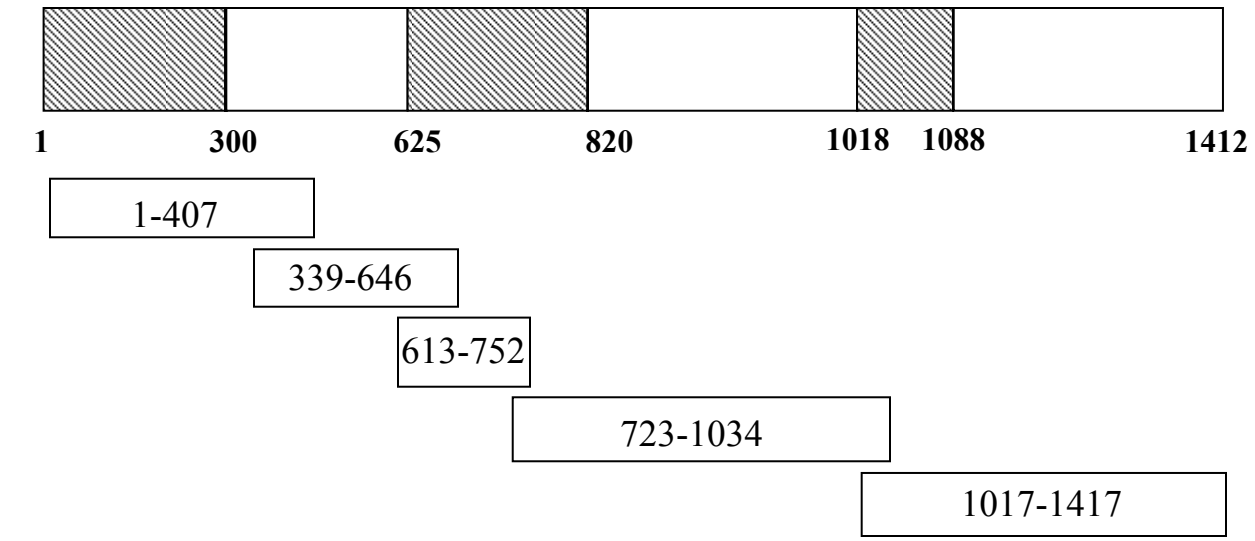


Figure 3. **AIB1 interacts non-specifically with the mouse immunoglobulin control.** Whole-cell extract from MCF-7 cells was immunoprecipitated with either a monoclonal BRCA1 antibody (AB2, Oncogene Science) or mouse immunoglobulin control (Upstate Biotechnology) and then immunoblotted with AIB1.

Figure 4



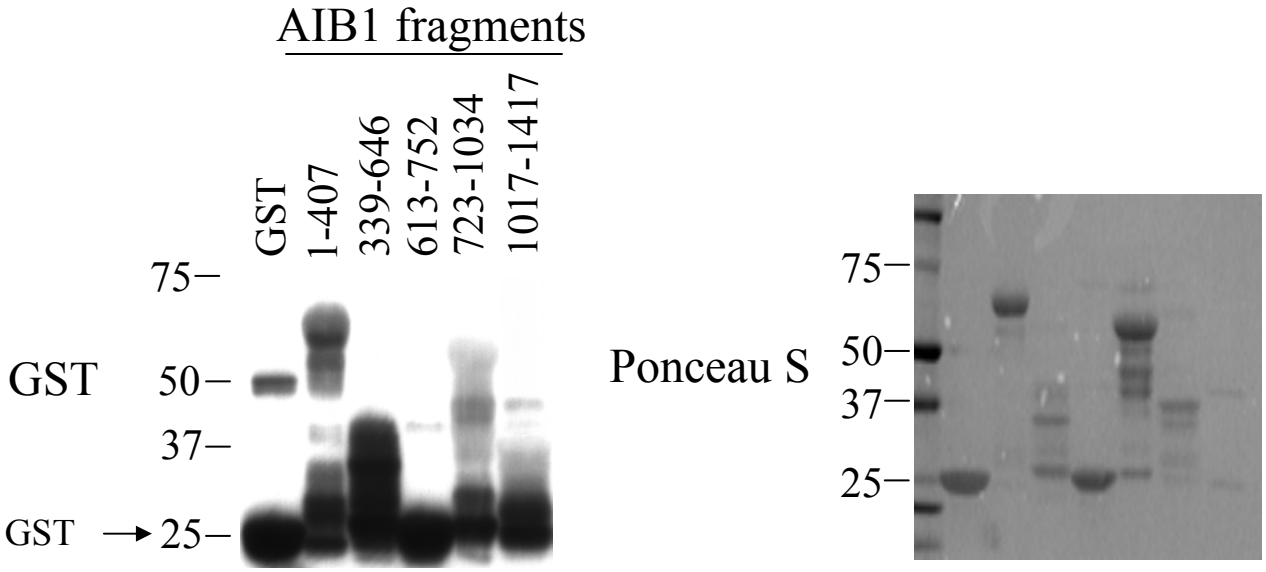
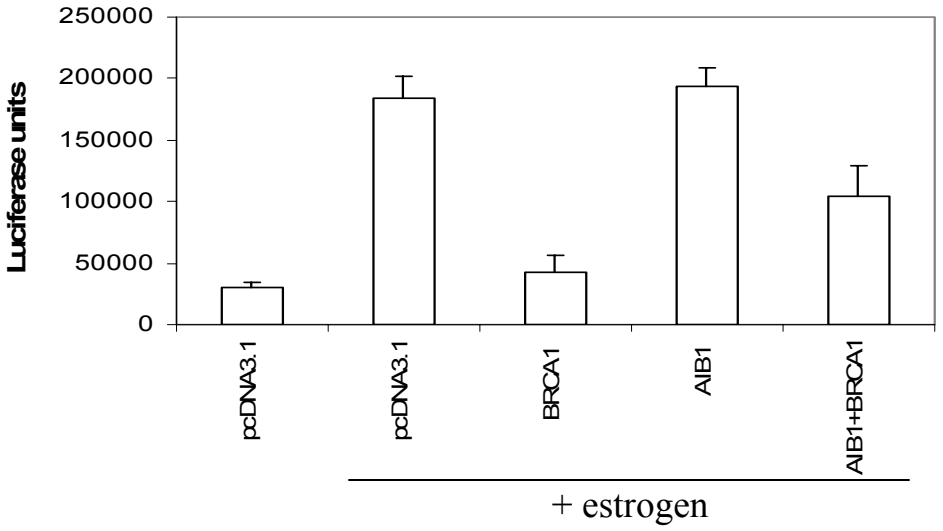


Figure 4. Diagram of AIB1 protein indicating the locations of GST-AIB1 fragments. GST-AIB1 fragments were produced in *E. coli* bacteria and purified with glutathione beads. The resulting fragments were analyzed by immunoblot with a monoclonal GST antibody (Santa Cruz Biotech) and by Ponceau S staining of total protein. There were some degradation products of the GST-AIB1 fragments.

Figure 5

A.



B.

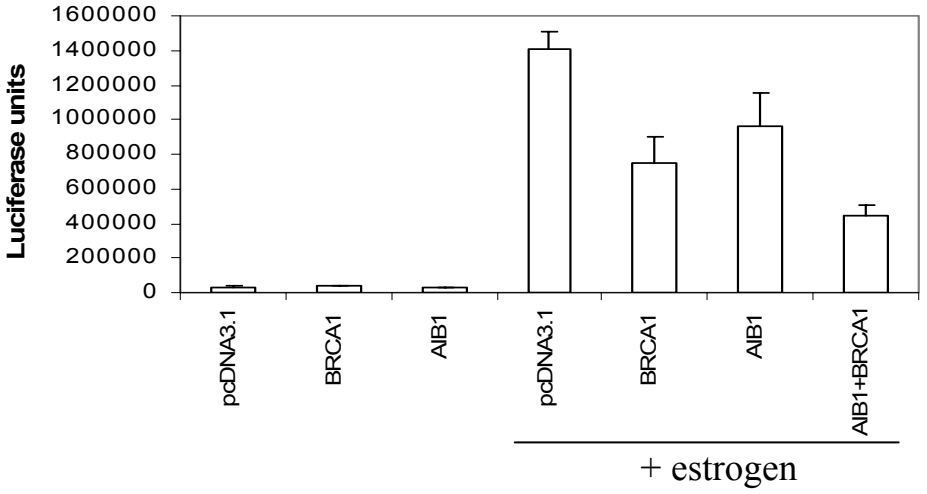


Figure 5. **Effect of transfected BRCA1 and AIB1 on ER transcriptional activity in MCF-7 and T47D breast cancer cells**. Either MCF-7 (A) or T47D (B) cells were plated in 5% charcoal-stripped serum in IMEM for 3 days prior to transfection. Cells were transfected with the following plasmids: pcDNA3.1, pcDNA3-AIB1, and pcDNA3-BRCA1. The ERE reporter construct was co-transfected, which consists of 3 copies of the estrogen responsive element regulating luciferase. After 24 hours of transfection, 100 nM of estrogen was added to the cells for another 24 hours. The cells were lysed and luciferase levels were quantified. Conditions are in triplicate.

Figure 6

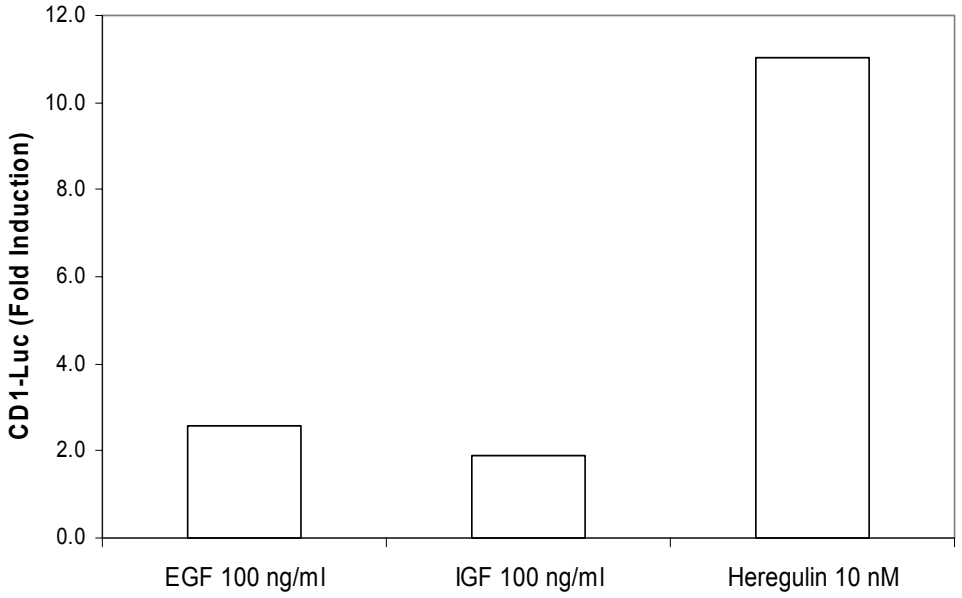


Figure 5. **Growth factors induce cyclin D1 promoter activity in MCF-7 cells**. MCF-7cells were plated in 10% fetal bovine serum in IMEM. After the cells were attached, the cells were transfected with a -1745 cyclin D1 promoter-luciferase reporter construct. The media was changed to serum-free IMEM for the transfection. After 24 hours of transfection, 100 ng/ml of EGF, 100 ng/ml IGF-1, and 10 nM of heregulin β was added to the cells for another 24 hours. The cells were lysed and luciferase levels were quantified. Conditions are in triplicate.

Figure 7

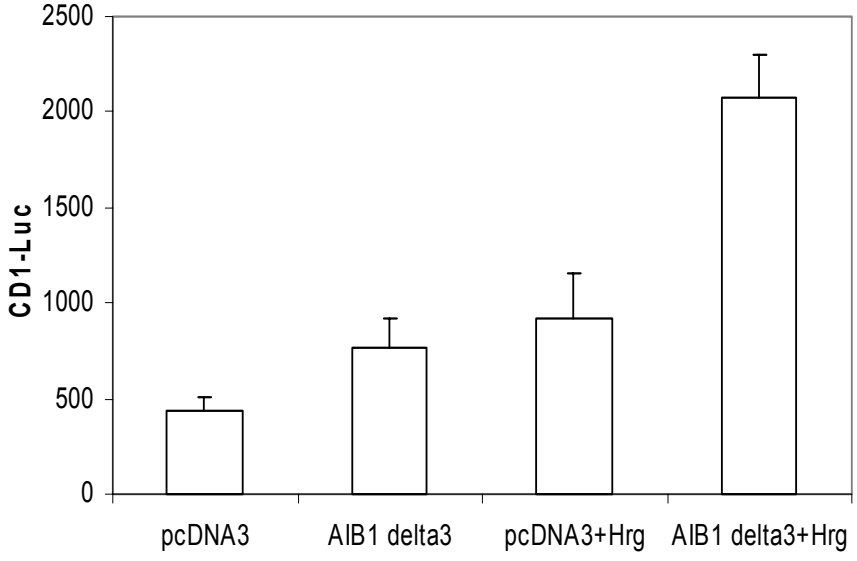


Figure 5. **AIB1-Δ3 enhances growth factor induced activation of the cyclin D1 promoter in BRCA1-deficient HCC1937 cells**. HCC1937 cells were plated in 10% fetal bovine serum in IMEM. After the cells were attached, the cells were transfected with a -1745 cyclin D1 promoter-luciferase reporter construct. The media was changed to serum-free IMEM for the transfection. After 24 hours of transfection, 10 nM of heregulin β (Hrg) was added to the cells for another 24 hours. The cells were lysed and luciferase levels were quantified. Conditions are in triplicate.

KEY RESEARCH ACCOMPLISHMENTS

- The experimental conditions for analyzing the AIB1 and BRCA1 interaction need to be modified.
- GST-AIB1 fragments have been purified to be used in pull-down assays with BRCA1.
- AIB1 was shown to partially reverse the BRCA1-mediated repression of ER transcriptional activity in MCF-7 breast cancer cells.
- Cyclin D1 promoter activity can be used as a read-out for growth factor induced gene expression.

REPORTABLE OUTCOMES

Abstracts:

Lahusen, J.T., Wellstein, A., and Riegel, A.T. AIB1 Regulates EGFR Phosphorylation and Activity in Cancer. Biomedical Sciences Research Fair (2006). Georgetown University, Washington, D.C.

Oh, A.S., Stoica, G.E., **Lahusen, J.T**., Wellstein, A., and Riegel, A.T. Functional Role of Tyrosine Phosphorylated AIB1/ACTR. Abstract No. 243. Keystone Symposia, Nuclear Receptor: Orphan Brothers. Banff, Alberta, Canada 2006

CONCLUSIONS

The AIB1 and BRCA1 interaction experiments are ongoing as outlined in **Task 1**. The interaction of AIB1 and BRCA1 that I observed in preliminary experiments needs to be verified with a different method due to non-specific binding of AIB1 in the immunoprecipitation. Also, as part of **Task 1**, AIB1 was able to partially reverse BRCA1 mediated repression of ER

transcriptional activity in MCF-7 breast cancer cells. I am currently testing other cell lines for the ER transcriptional assays. To address **Task 2**, AIB1-Δ3 and growth factors were shown to enhance cyclin D1 promoter activity, which could be used as a model system for testing the functional relationship of AIB1 and BRCA1 in growth factor-dependent gene expression.

ABBREVIATIONS

AIB1- Amplified in breast cancer 1
bHLH- basic helix-loop-helix
BRCA1- Breast Cancer Susceptibility Protein
CCS- Charcoal stripped calf serum
EGF- Epidermal Growth Factor
ERα- Estrogen receptor alpha
ERE-TK-Luc- estrogen responsive element-thymidine kinase-luciferase
GST- Glutathione-S-Transferase
IGF- Insulin-like Growth Factor I
IMEM- Iscove's Modified Eagle Medium
PAS- Per-Arnt-Sim
PR- Progesterone receptor
SRC- Steroid Receptor Coactivator

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